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(54) Title: IMPROVEMENT IN IONIC RESERVOIR THROUGH APPLICATION OF AN ELECTRICAL POTENTIAL

(57) Abstract

The present invention provides an improved membrane based biosensor comprising a lipid membrane incorporating ionophores the conductivity of the membrane being dependent on the presence or absence of an analyte, a reference electrode, a sensing electrode on to which is deposited the lipid membrane such that a functional reservoir exists between the lipid membrane and the sensing electrode. The improvement comprises including in the biosensor means to apply a dc electrical potential offset to the sensing electrode relative to the reference electrode.

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IMPROVEMENT IN IONIC RESERVOIR THROUGH APPLICATION OF AN ELECTRICAL POTENTIAL

The present invention relates to an improved membrane based biosensor and to a method of improving the performance of membrane based biosensors.

Biosensors based on ion channels or ionophores contained within lipid membranes that are deposited onto metal electrodes, where the ion channels are switched in the presence of analyte molecules have been described in International Patent Application Nos WO 92/17788, WO 93/21528, WO 94/07593 and US 5,204,239 (the disclosures of which are incorporated herein by reference). As these biosensors rely on changes in ion conduction through the membrane, usually mediated by an ionophore, it is important that there exists an ionic reservoir between the electrode and the lipid membrane. Ideally this ionic reservoir between the electrode and the lipid reservoir is not totally depleted or filled, by conduction through the ionophore, during the course of the measurement cycle. The usual method of measuring the conductance changes is the use of alternating current (AC) impedance spectroscopy. The abovementioned disclosures have shown that good reservoirs can be produced using special linker lipid compounds.

The present inventors have now found that the application of a direct current (dc) potential offset superimposed onto the AC impedance signal can influence the apparent conduction of ions by the ionophore through the membrane. Without wishing to be bound by scientific theory it is believed that this modification of the ionophore conduction occurs through the modulation of the reservoir capacity and improvement in the reservoir homogeneity. This improvement in conduction of ions by the ionophore therefore allows the use of less ionophore which may be useful in producing more sensitive sensor membranes as less analyte is required to switch the ionophore on/off. A negative dc potential applied to the metal

electrode has been shown to improve the ion conduction by ionophores, whereas a positive dc potential applied to the metal electrode has been shown to decrease and even negate the apparent conduction of the ionophores through the membrane. This effect is especially noticeable when membranes are formed containing phosphatidyl choline based lipids. The inventor has found that by controlling the dc offset, the reproducibility of the ionophore conduction is greatly improved.

Accordingly, in a first aspect the present invention consists in an improved membrane based biosensor comprising a lipid membrane incorporating ionophores the conductivity of the membrane being dependent on the presence or absence of an analyte, a reference electrode, a sensing electrode onto which is deposited the lipid membrane such that a functional reservoir exists between the lipid membrane and the sensing electrode, the improvement comprising including in the biosensor means to apply a dc electrical potential offset to the sensing electrode relative to the reference electrode.

In a second aspect the present invention consists in an improved method of detecting the presence or absence of an analyte in a sample using a membrane based biosensor comprising a lipid membrane incorporating ionophores the conductivity of the membrane being dependent on the presence or absence of the analyte, a reference electrode, a sensing electrode on to which is deposited the lipid membrane such that a functional reservoir exists between the lipid membrane and the sensing electrode, the improvement comprising applying a dc electrical potential offset to the sensing electrode relative to the reference electrode.

In a third aspect by incorporating ionisable, polarisable, dipolar or otherwise electroactive species within the membrane based biosensor comprising a lipid membrane incorporating ionophores the conductivity of the membrane being dependent on the presence or absence of an analyte, a reference electrode, a sensing electrode onto which is deposited the lipid

membrane such that a functional reservoir exists between the lipid membrane and the sensing electrode, the appropriate dc potential can be induced between the sensor electrode and the analyte solution.

Although it is envisaged that generally it is preferred to apply a negative potential onto the metal sensor electrode in order to improve the ionophore conduction, it may be useful in some circumstances to apply a positive potential onto the metal sensor electrode thus reducing or negating the apparent ionophore conduction through the membrane.

In a preferred embodiment of the present invention a dc potential of between +500mV to -500mV is applied to the sensing electrode.

In a further preferred embodiment the dc offset is produced through the use of a counter electrode where the electrochemical potential between the counter electrode and the sensing electrode produces an electrical potential of between 0 to -500mV, with the sensing electrode being at the negative potential.

In a preferred embodiment the counter electrode is made from stainless steel.

In a further preferred embodiment the counter electrode is made from titanium.

In a further preferred embodiment the counter electrode is made from silver, gold, platinum, palladium, copper, chromium or molybdenum.

In another preferred embodiment the counter electrode is made from metals that are capable of being deposited in a thin film onto a plastic, glass or silicon substrate, said metals being stable for at least 30 minutes in aqueous solution and sets up the appropriate electrochemical potential relative to the sensing electrode on addition of an aqueous solution.

In a further preferred embodiment of the present invention the counter electrode is an electrochemically neutral metal relative to the sensing electrode and the dc electrical potential of between +500 to -500mV is created by electronic means.

In a further preferred embodiment of the present invention the counter electrode produces an electrochemical potential relative to the sensing electrode which is enhanced or negated or reversed using a dc electrical potential created by electronic means to give a potential of between +500 to -500mV.

In yet another preferred embodiment of the present invention, the dc offset potential at the sensing electrode, onto which is deposited the lipid membrane, is controlled using a three terminal measurement, where the impedance measurement is made between the counter electrode and the working electrode which is the sensing electrode and where the dc offset potential is controlled by a reference electrode to be between +500 to -500mV as required.

The metals used for the counter electrode and the reference electrode in the three terminal measurement may be any of the commonly used metals and electrode combinations commonly used in these measurements as known to those skilled in the art.

In a further preferred embodiment of the present invention the metal used for the sensing electrode is a layer of freshly evaporated or sputtered gold. Alternatively, a freshly cleaned gold surface, which can be produced using plasma etching or ion-beam milling, can be used.

It is further preferred that the first layer of the lipid membrane is produced using the linker lipid shown in figure 1, the disulfide of mercaptoacetic acid, linker gramicidin shown in figure 2, the membrane spanning lipid (C) and the membrane spanning lipid (D) both shown in figure 3.

It is further preferred that the second layer of the lipid membrane is produced from diphytanyl phosphatidyl choline, glycerol diphytanyl ether, shown in figure 7, and biotinylated gramicidin shown in figure 4.

In a further preferred embodiment the second layer lipid contains at least a proportion of a phosphatidyl choline, or phophatidyl ethanolamine or phosphatidic acid lipid.

In a further preferred embodiment the second layer lipid contains at least a proportion of a charged lipid.

In a further preferred embodiment the lipid membrane is a monolayer.

As will be appreciated by those skilled in the art, if the sensing of an analyte occurs through the switching off or on of an ionophore contained within the lipid sensing membrane on addition of analyte, then it is possible to monitor this change in conduction by measuring the amount of electrical potential required in order to maintain the membrane conduction value at the initial ungated membrane conduction value. The magnitude and sign of the electrical potential is then related to the amount of analyte present in the sample.

By increasing the signal spectral inhomogeneity the information content in the signal can be increased with the consequent possibility of improved signal to noise. One mechanism for achieving this is to take advantage of the system voltage dependence by applying a non sinusoidal excitation and then analysing the results by fourier transform in which case the signal information content will be increased due to the cross modulation products in the output.

By automatically selecting a dc potential the sensitivity can be optimised. This may sometimes require the use of a calibrating dose of analyte for each measurement. (See Example 2 as a means of minimising drift.)

The present invention also provides an improved method for detecting response to an analyte in which a signal may derived by altering and monitoring dcbias potential, while analyte is binding to the channels during the biosensor gating event, either to maintain the admittance

constant preferably at the frequency for minimum phase or similarly to maintain the phase constant preferably at the frequency for minimum phase.

The present invention further provides an improved method for detecting the electrode response to analyte in which the signal response is optimised by automatically altering the dc bias potential to obtain maximum sensitivity or minimum drift.

In order that the nature of the present invention may be more clearly understood the invention will now be described by way of non-limiting example.

Example 1

On a clean glass or plastic slide, an adhesion layer of chromium (50 angstrom) followed by a gold layer (200-2000 angstroms) is evaporated. The freshly evaporated gold coated electrode is taken and immediately immersed in an ethanolic solution of linker lipid (Fig 1) (300ul of 10mM), the disulfide of mercaptoacetic acid (150ul of 10mM), linker gramicidin (Fig 2) (150ul of 0.01 mg/ml), membrane spanning lipid C (Fig 3)(2.25 ul of 0.1 mM) and membrane spanning lipid D (Fig 3) (45ul of 1 mM) in ethanol (50 ml). The gold coated electrode is left immersed in the solution for 5-60 minutes, rinsed with ethanol and assembled into a teflon slide assembly holder such that an electrode surface is defined by a circular teflon well pressed onto the gold electrode. The teflon well forms a tight, water impermeable seal at the electrode perimeter. This procedure forms the first layer of the bilayer sensor membrane and may be stored in ethanol, glycerol, ethylene glycol or other alcohol for several months. Formation of the second layer of the bilayer membrane is carried out by addition of 5ul of a solution containing 14mM of diphytanyl phosphatidyl choline/glyceryl diphytanyl ether (7:3 ratio), biotinylated gramicidin (Fig 4) in a ratio of 100,000:1 (total lipid):gramicidin. The well assembly was then rinsed twice with phosphate buffered saline (PBS) resulting the formation of the second lipid layer of the bilayer sensing membrane. The well assembly holds approximately 150ul of

PBS. Into this 150 ul of PBS in the well is placed a counter electrode, a connection is made between this counter electrode and the impedance bridge measuring apparatus. To complete the electrical circuit, the other connection is made between the gold electrode and the impedance bridge. In order to control the dc potential offset a reference electrode is inserted into the well also contacting the PBS solution and the potential is controlled such that the gold electrode potential may be varied. The apparatus needed to make such three terminal measurements are known to those skilled in the art. Alternatively, the dc offset may be varied by changing the metal type which makes up the counter electrode. This sets up electrochemical potential between the counter electrode and the gold electrode. A dc offset may also be produced electronically in a two terminal measurement. Using the impedance bridge the conduction of the membrane may then be determined. Standard Bode plots are shown in figure 5. The effect of changing the counter electrode material, thus changing the potential, on gramicidin induced membrane conduction is shown. As can be seen stainless steel and titanium counter electrodes produce more conductive membranes than silver or gold counter electrodes when equivalent membrane sensor electrodes are measured.

Using a three terminal measurement it was found that the gramicidin induced membrane conduction increases as a negative potential is applied to the sensor membrane in the range of between 0mV to -500mV. Figure 6 shows the effect of varying the potential on gramicidin containing membranes. As an indication of conduction the frequency at phase minimum is used. The higher the frequency at phase minimum, the more conductive the membrane. However, on application of a positive potential (0mV to +500mV) relative to the gold electrode the gramicidin induced membrane conduction decreased, such that at +200mV the membrane was ionically insulating.

It is believed that using counter electrode metals such as stainless steel or titanium places a dc offset of between -150mV to -400mV on the gold electrode relative to the counter electrode. It has been further found that the reproducibility in terms of conduction for a particular concentration of ionophore in the membrane has been improved from coefficients of variation (cv's) of 30-60% using silver counter electrodes to cv's of 10-15% using stainless steel electrodes.

Similar effects were found if ionophores such as valinomycin were used instead of the gramicidin derivatives.

Example 2

A membrane was formed as described above in Example 1. A dc offset across the biosensor membrane was established using a three terminal voltage clamp with a platinum counter electrode, a silver chloride reference electrode and a gold sensing electrode.

Figure 10 shows the effect of varying the dc offset on the drift in the biosensor output. The output signal was the frequency at minimum phase. The graph Y axis shows the rate of the frequency at minimum phase divided by the initial frequency at minimum phase.

The RC network in Figure 10 is a represntation of the passive electrical properties of the sensor membrane. This model consists of two capacitors connected in series, one with a value of 0.1 microFarad the second with a value of 0.01 microFarad and a resistor of about 300 kilOhm connected in parallel with the 0.01 microFarad capacitor. When this network was connected to the measuring apparatus the intrinsic drift in the apparatus was found to be negligible as is indicated in Fig 10.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the

invention as broadly described, the present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Claims

1. An improved membrane based biosensor comprising a lipid membrane incorporating ionophores the conductivity of the membrane being dependent on the presence or absence of an analyte, a reference electrode, a sensing electrode on to which is deposited the lipid membrane such that a functional reservoir exists between the lipid membrane and the sensing electrode, the improvement comprising including in the biosensor means to apply a dc electrical potential offset to the sensing electrode relative to the reference electrode.

2. An improved membrane based biosensor according to claim 1, wherein the means to apply a dc electrical potential is a means capable of applying a dc electrical potential of between +500mV to -500mV to the sensing electrode.

3. An improved membrane based biosensor according to claim 1 or 2, wherein the dc offset is produced by a counter electrode.

4. An improved membrane based biosensor according to claim 3, wherein the electrochemical potential between the counter electrode and the sensing electrode produces an electrical potential of between 0 to -500mV, with the sensing electrode being at the negative potential.

5. An improved membrane based biosensor according to claim 3 or 4, wherein the counter electrode is made from stainless steel.

6. An improved membrane based biosensor according to claim 3 or 4, wherein the counter electrode is made from titanium.

7. An improved membrane based biosensor according to claim 3 or 4, wherein the counter electrode is made from silver, gold, platinum, palladium, copper, chromium or molybdenum.

8. An improved membrane based biosensor according to claim 3 or 4, wherein the counter electrode is made from a metal that is capable of being deposited in a thin film on to a plastic, glass or silicon substrate, said metal being stable for at least 30 minutes in aqueous solution and sets up the appropriate electrochemical potential relative to the sensing electrode on addition of an aqueous solution.

9. An improved membrane based biosensor according to any one of claims 3 to 8, wherein the counter electrode is an electrochemically neutral metal relative to the sensing electrode and the dc electrical potential of between +500mV to -500mV is created by electronic means.

10. An improved membrane based biosensor according to any one of claims 3 to 8, wherein the counter electrode produces an electrochemical potential relative to the sensing electrode which is enhanced or negated or reversed using a dc electrical potential created by electronic means to give a potential of between +500mV to -500mV.

11. An improved membrane based biosensor according to any one of claims 3 to 10, wherein the dc offset potential at the sensing electrode, onto which is deposited a lipid membrane, is controlled using a three terminal measurement, wherein the impedance measurement is made between the counter electrode and the working electrode which is the sensing electrode and where the dc offset potential is controlled by a reference electrode to be between +500mV to -500mV as required.

12. An improved membrane based biosensor according to any one of claims 3 to 11, wherein the sensing electrode comprises a metal

13. An improved membrane based biosensor according to claim 12, wherein the metal used for the sensing electrode is a layer of freshly evaporated, sputtered, plasma etched or ion beam milled gold.

14. An improved membrane based biosensor according to any one of claims 3 to 13, wherein the lipid membrane comprises a first layer of linker lipid (Fig 1), the disulfide of mercaptoacetic acid, linker gramicidin (Fig 2), membrane spanning lipid C (Fig 3) and membrane spanning lipid D (Fig 3).

15. An improved membrane based biosensor according to claim 14, wherein the lipid membrane comprises a second layer of diphytanyl phosphatidyl choline, glycerol diphytanyl ether, and biotinylated gramicidin (Fig 4).

16. An improved membrane based biosensor according to claim 15, wherein the said second layer contains at least a proportion of a phosphatidyl choline, or phosphatidyl ethanolamine or phosphatidic acid lipid.

17. An improved membrane based biosensor according to claims 15 or 16, wherein the said second layer contains at least a proportion of a charged lipid.

18. An improved membrane based biosensor according to any one of claims 1 to 13, wherein the lipid membrane is a monolayer.

19. An improved method of detecting the presence or absence of an analyte in a sample using a membrane based biosensor comprising a lipid membrane incorporating ionophores the conductivity of the membrane being dependent on the presence or absence of the analyte, a reference electrode, a sensing electrode on to which is deposited the lipid membrane such that a functional reservoir exists between the lipid membrane and the sensing electrode, the improvement comprising applying a dc electrical potential offset to the sensing electrode relative to the reference electrode.

20. An improved method according to claim 19, wherein a dc electrical potential of between +500mV to -500mV is applied to the sensing electrode.

21. An improved method according to claim 19 or 20, wherein the dc offset is produced by a counter electrode.

22. An improved method according to claim 21, wherein the electrochemical potential between the counter electrode and the sensing electrode produces an electrical potential of between 0 to -500mV, with the sensing electrode being at the negative potential.

23. An improved method according to claim 21 or 22, wherein the counter electrode is made from stainless steel.

24. An improved method according to claim 21 or 22, wherein the counter electrode is made from titanium.

25. An improved method according to claim 21 or 22, wherein the counter electrode is made from silver, gold, platinum, palladium, copper, chromium or molybdenum.

26. An improved method according to claim 21 or 22, wherein the counter electrode is made from a metal that is capable of being deposited in a thin film on to a plastic, glass or silicon substrate, said metal being stable for at least 30 minutes in aqueous solution and sets up the appropriate electrode chemical potential relative to the sensing electrode on addition of an aqueous solution.

27. An improved membrane based biosensor according to any one of claims 21 to 26, wherein the counter electrode is an electrochemically neutral metal relative to the sensing electrode and the dc electrical potential of between +500mV to -500mV is created by electronic means.

28. An improved membrane based biosensor according to any one of claims 21 to 26, wherein the counter electrode produces an electrochemical potential relative to the sensing electrode which is enhanced or negated or reversed using a dc electrical potential created by electronic means to give a potential of between +500mV to -500mV.

29. An improved membrane based biosensor according to any one of claims 21 to 28, wherein the dc offset potential at the sensing electrode, onto which is deposited a lipid membrane, is controlled using a three terminal measurement, wherein the impedance measurement is made between the counter electrode and the working electrode which is the sensing electrode and where the dc offset potential is controlled by a reference electrode to be between +500mV to -500mV as required.

30. An improved membrane based biosensor according to any of claims 21 to 29, wherein the sensing electrode comprises metal.

31. An improved method according to claim 30, wherein the metal used for the sensing electrode is a layer of freshly evaporated, sputtered, plasma etched or ion beam milled gold.

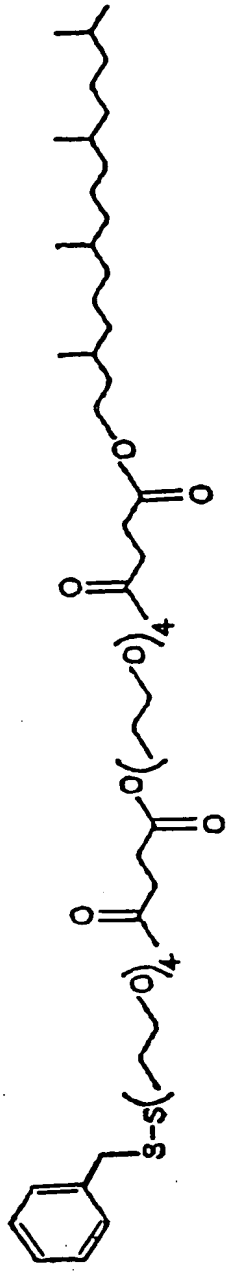
32. An improved method according to any one of claims 21 to 31, wherein the lipid membrane comprises a first layer of linker lipid (Fig 1), the disulfide of mercaptoacetic acid, linker gramicidin (Fig 2), membrane spanning lipid C (Fig 3) and membrane spanning lipid D (Fig 3).

33. An improved method according to claim 32, wherein the lipid membrane comprises a second layer diphytanyl phosphatidyl choline, glycerol diphytanyl ether, and biotinylated gramicidin (Fig 4).

34. An improved membrane based biosensor according to claim 33, wherein the said second layer contains at least a proportion of a phosphatidyl choline, or phosphatidyl ethanolamine or phosphatidic acid lipid.

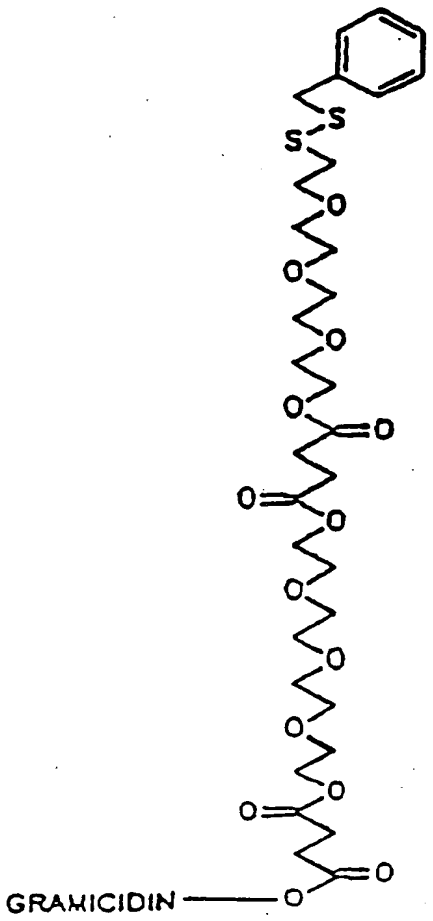
35. An improved membrane based biosensor according to claim 33 or 34, wherein the said second layer contains at least a proportion of a charged lipid.

36. An improved membrane based biosensor according to any one of claims 19 to 31, wherein the sensing membrane is a monolayer.



Linker Lipid

FIGURE 1



Linker Gramicidin

FIGURE 2

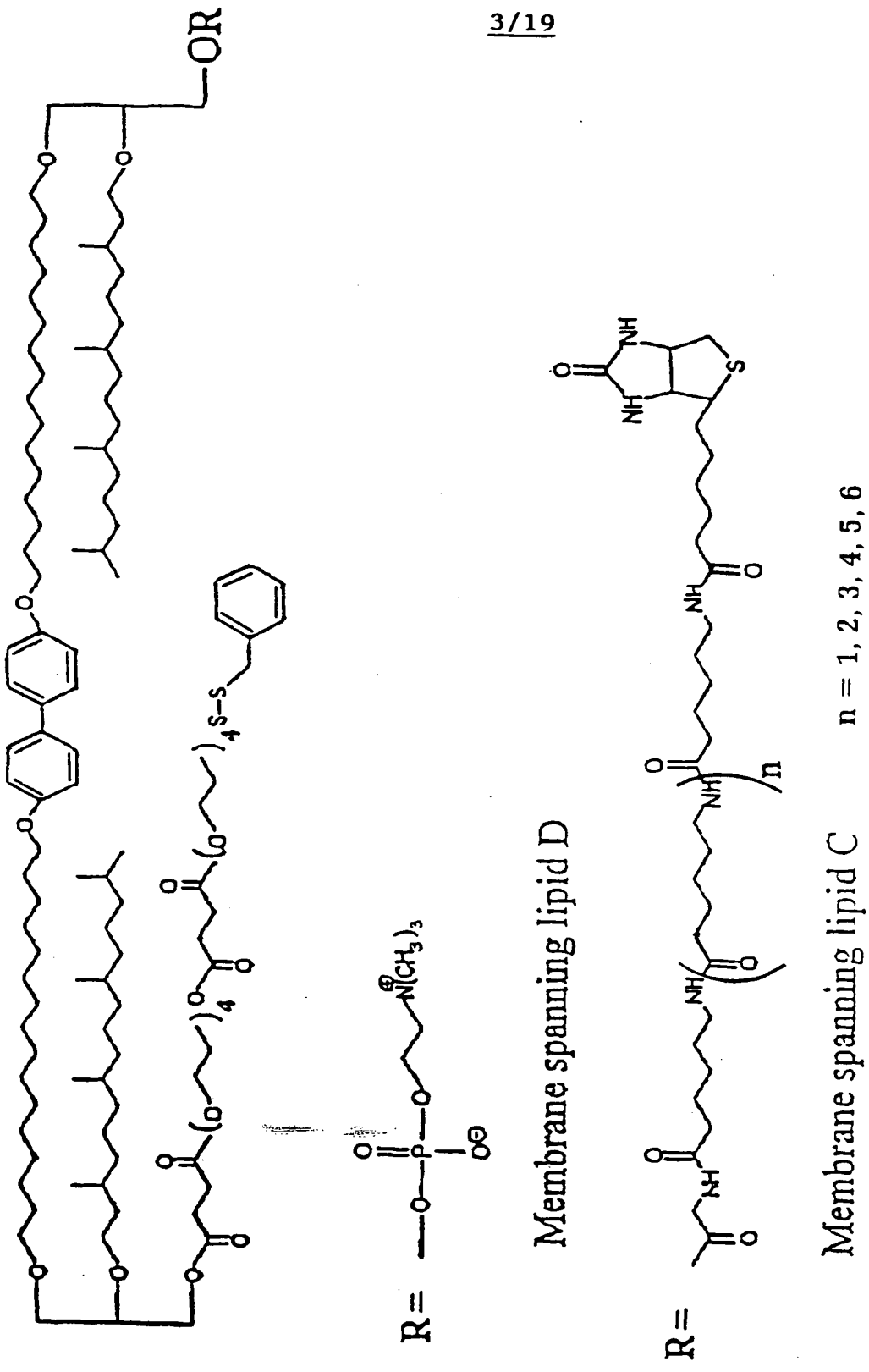
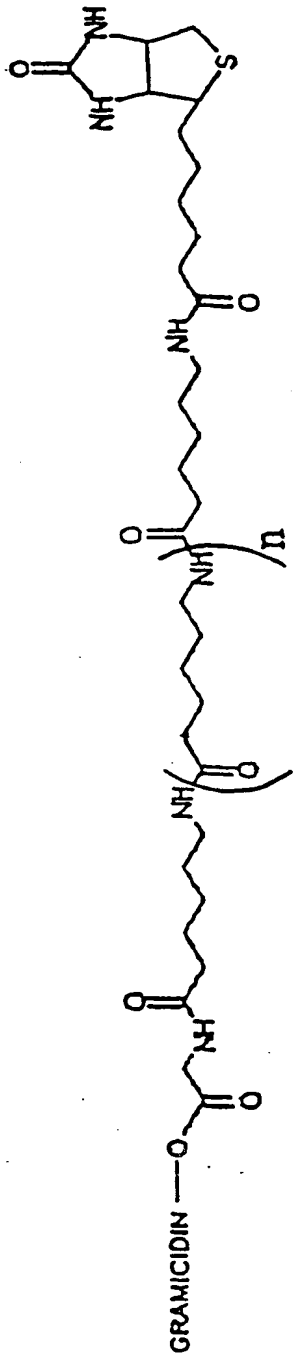


FIGURE 3



Biotinylated gramicidin

FIGURE 4

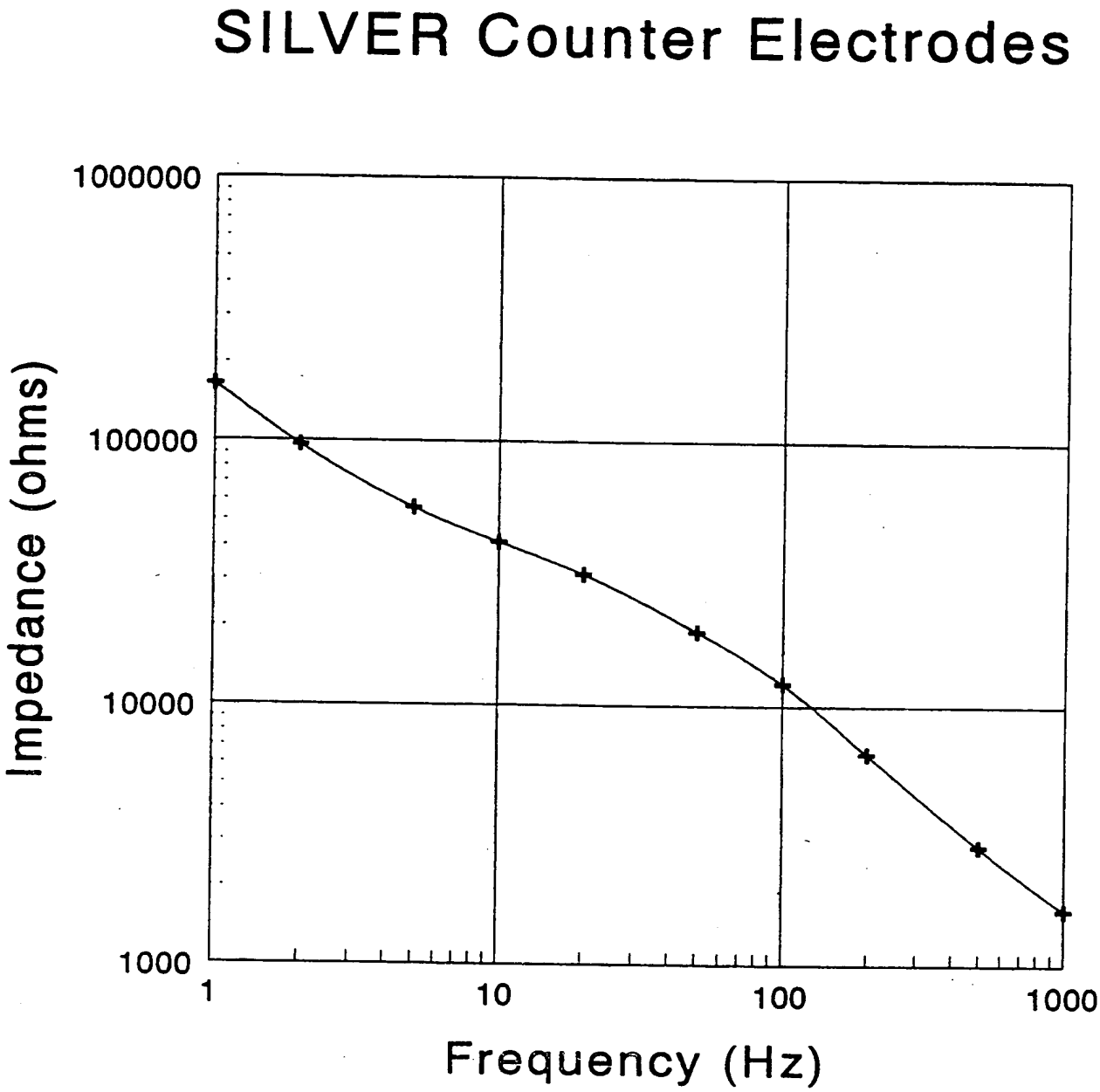


FIGURE 5A

SILVER Counter Electrodes

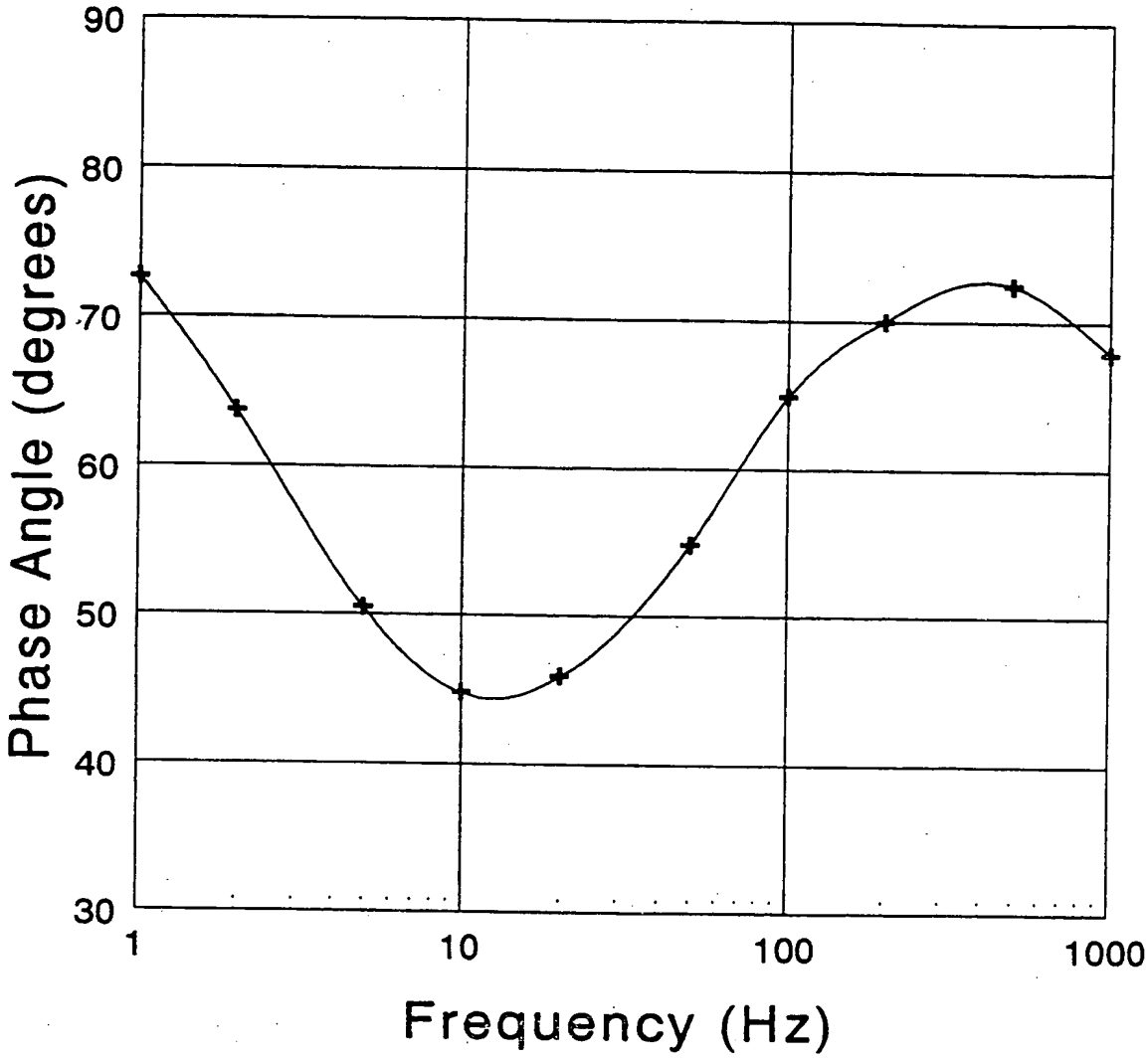


FIGURE 5B

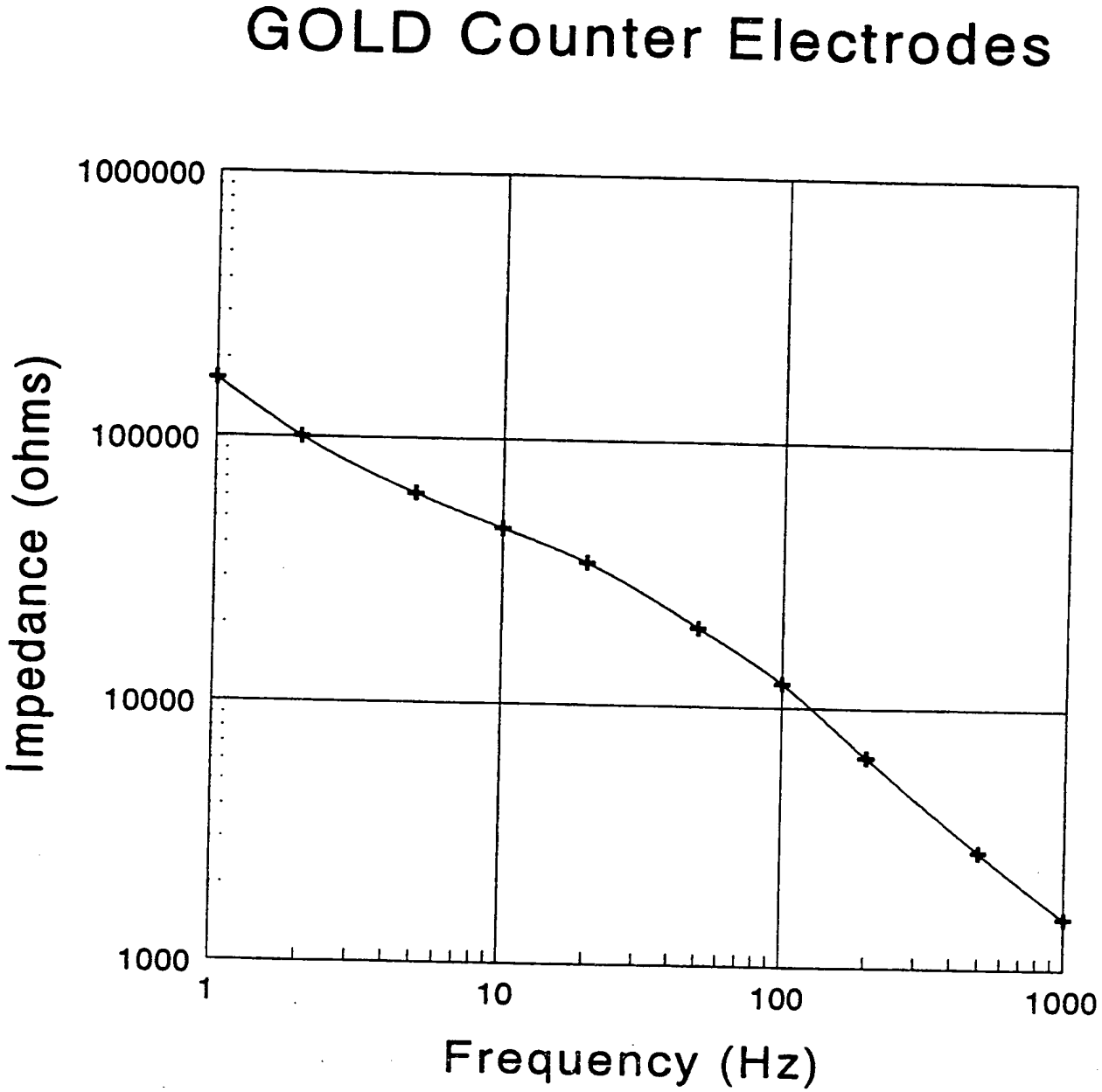


FIGURE 5C

GOLD Counter Electrodes

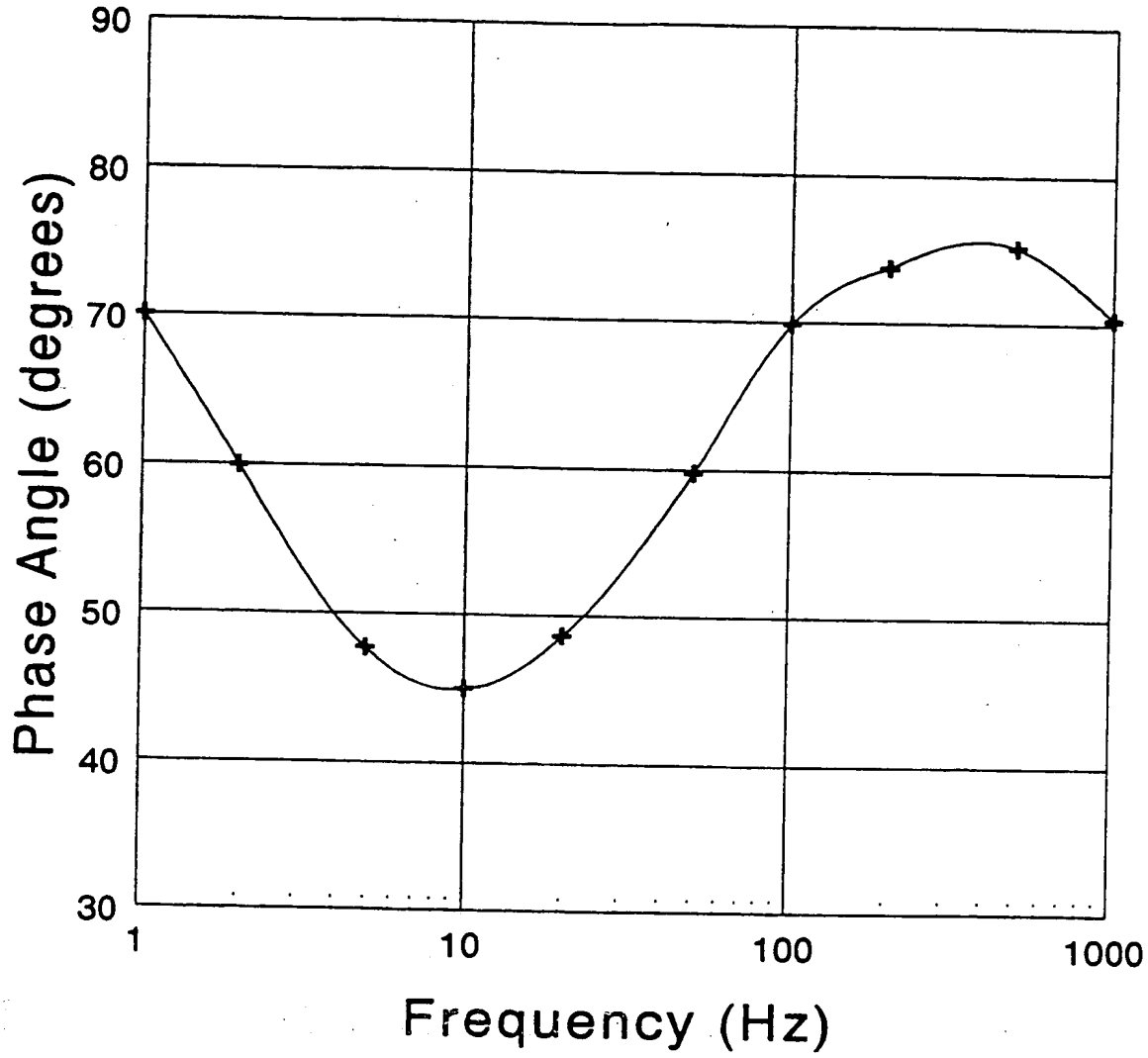


FIGURE 5D

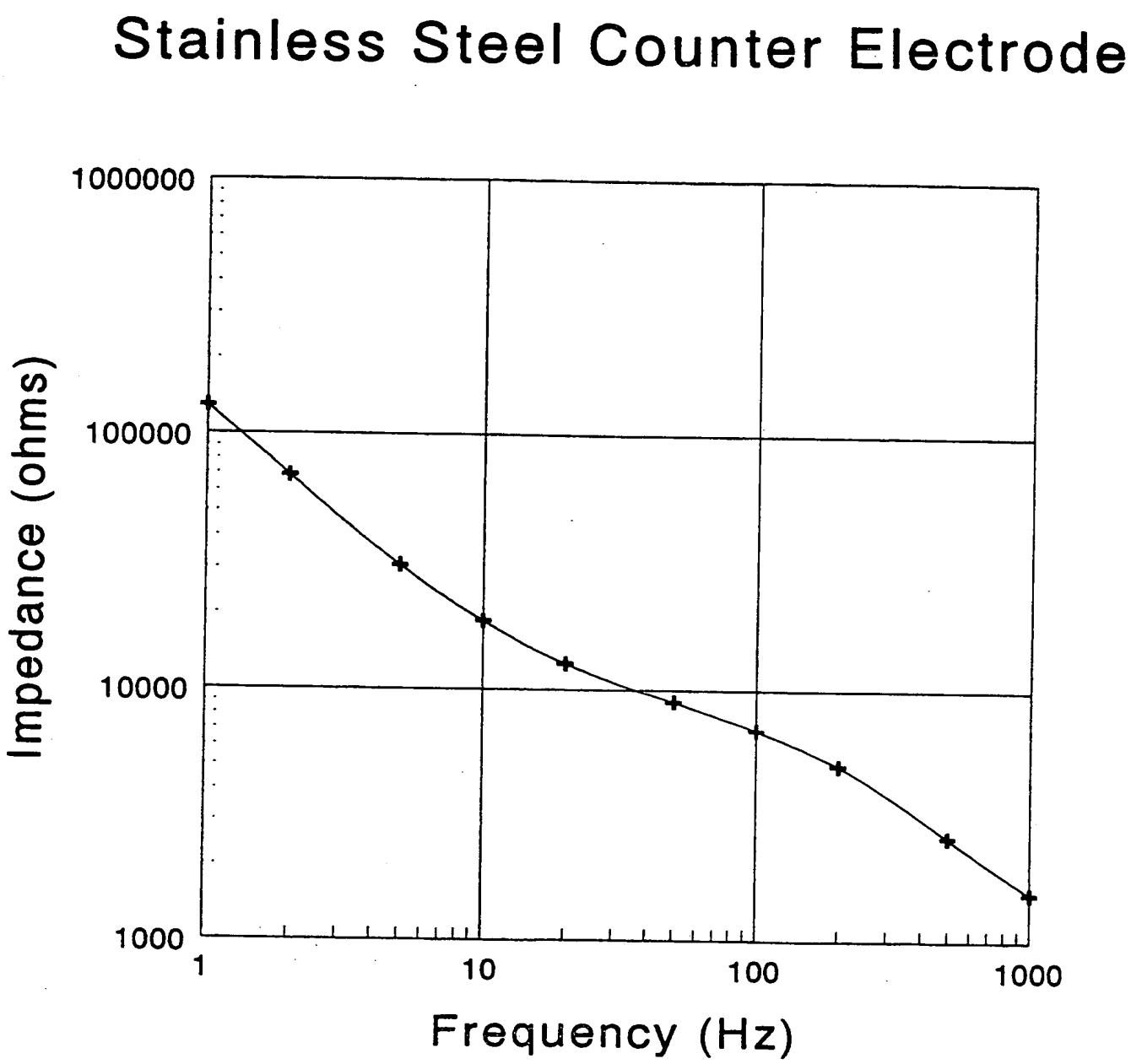


FIGURE 5E

Stainless Steel Counter Electrode

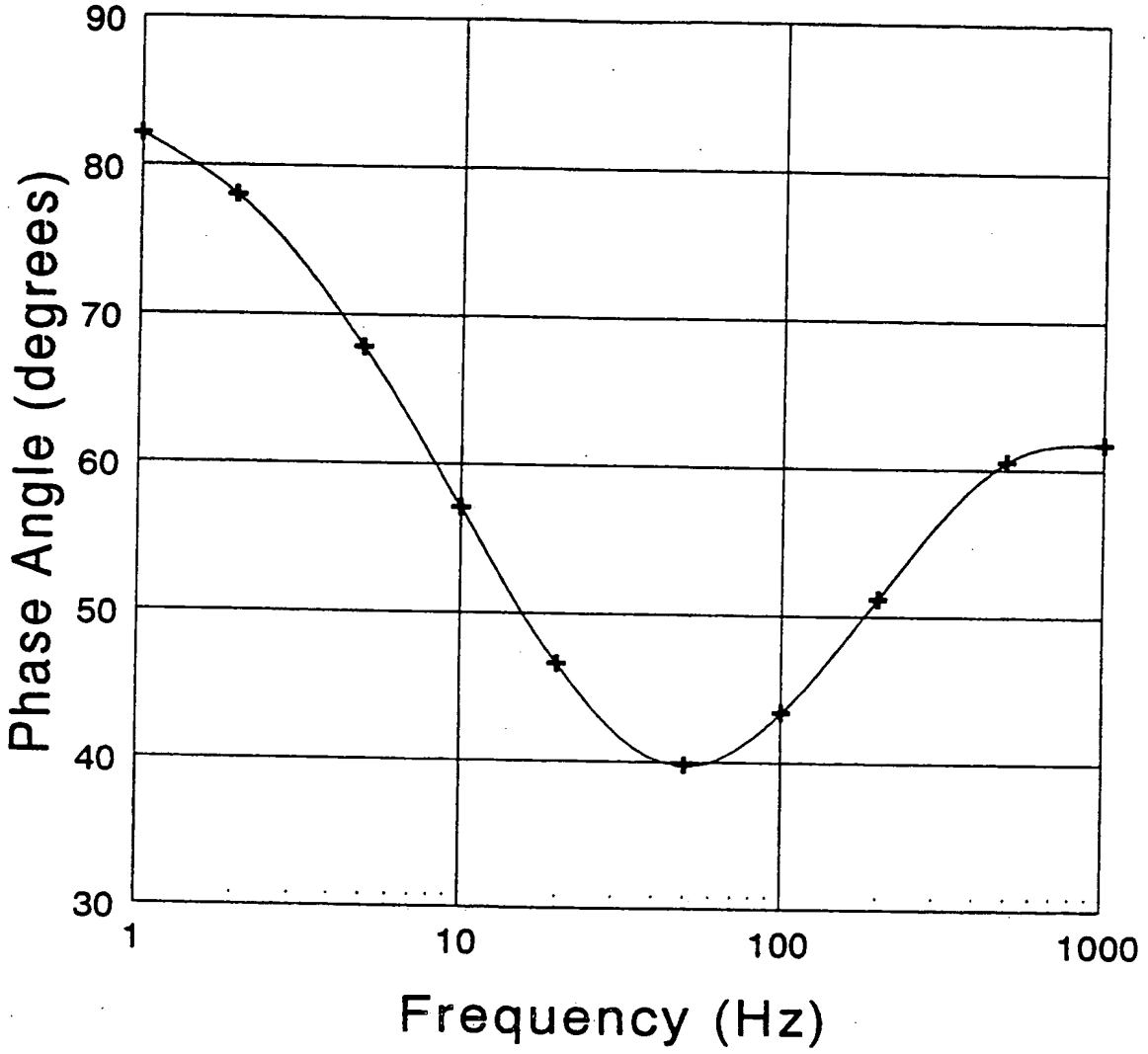


FIGURE 5F

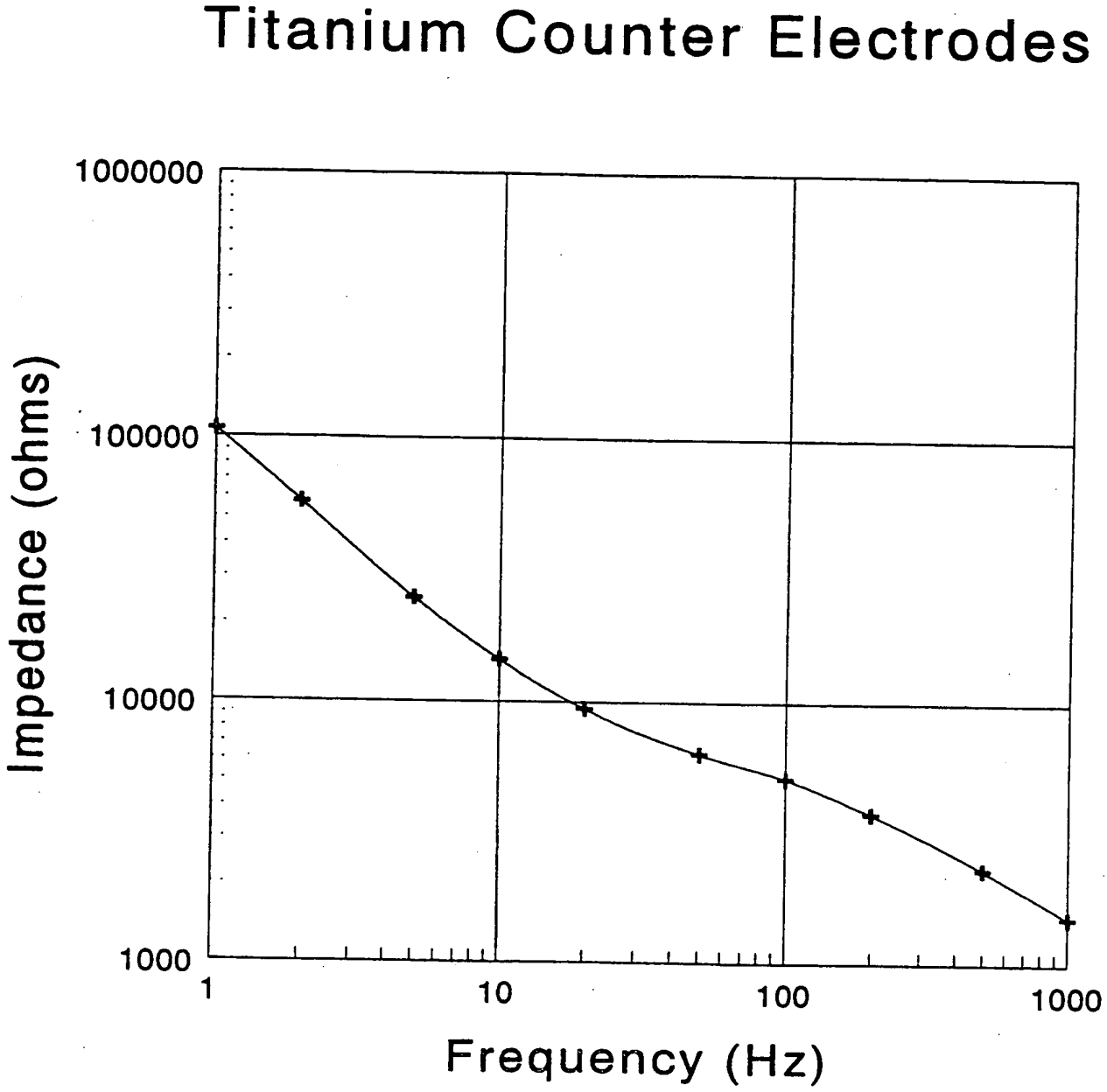


FIGURE 5G

Titanium Counter Electrodes

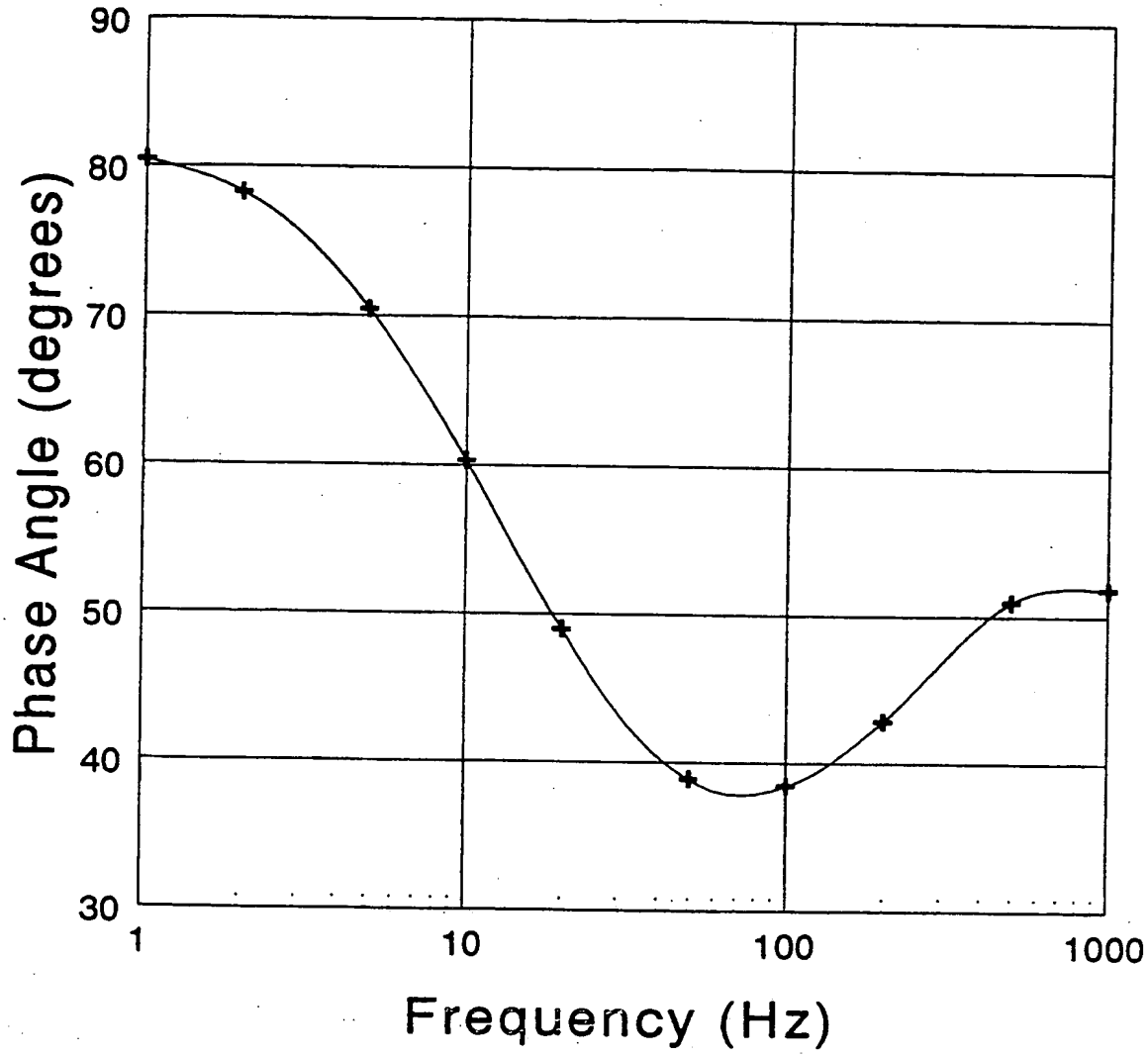


FIGURE 5H

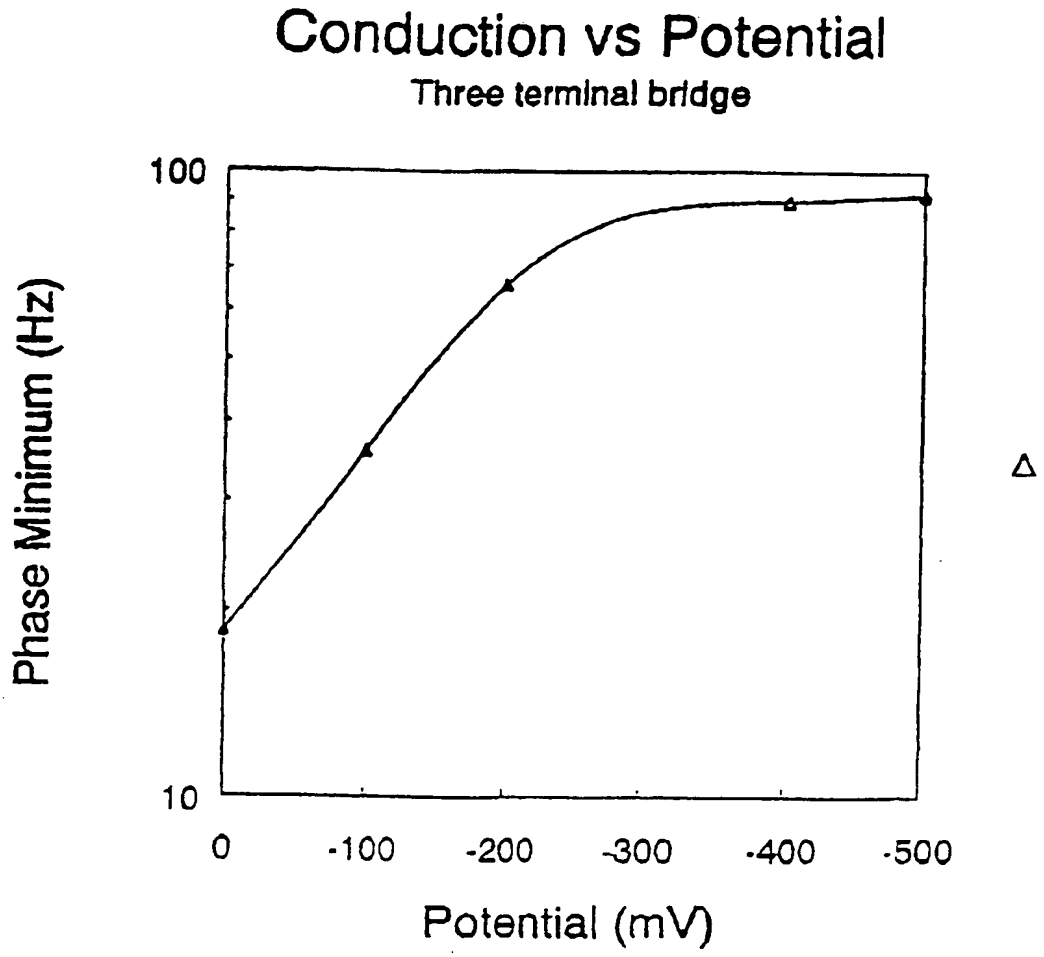
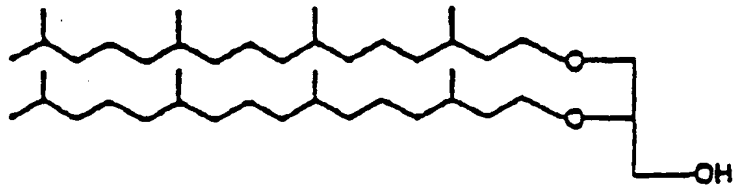
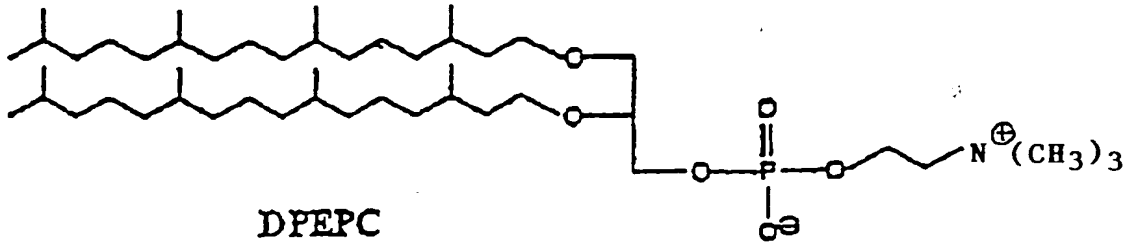


FIGURE 6



GDPE



DPEPC

FIGURE 7

X 1

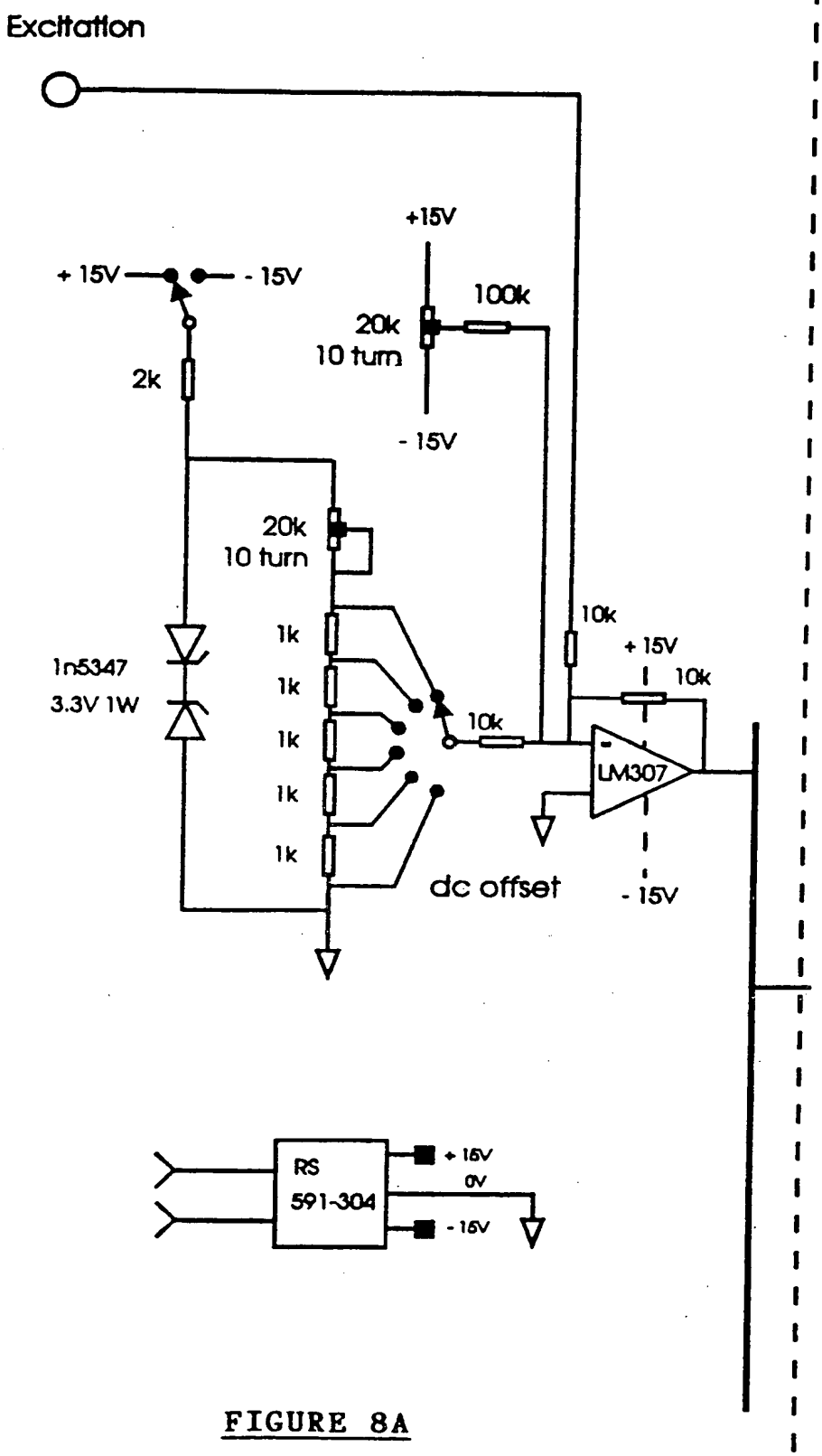
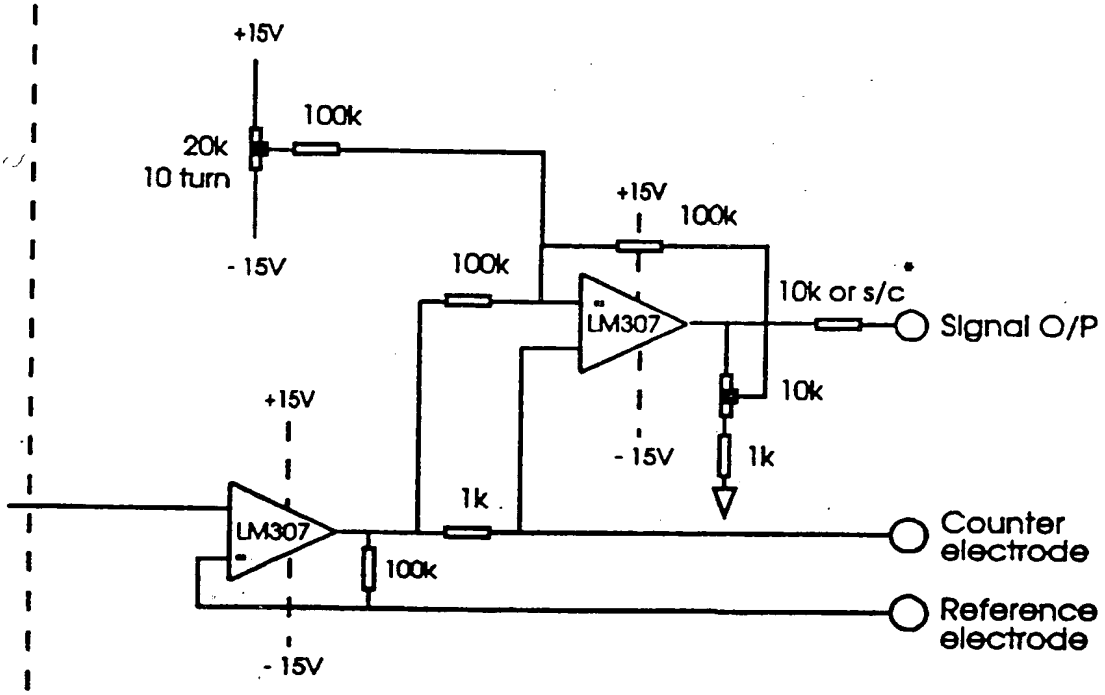


FIGURE 8A

X 16



* S/C for use with voltmeters or AMLAB'S
10k for use with ammeters or AMBRI bridg s

FIGURE 8B

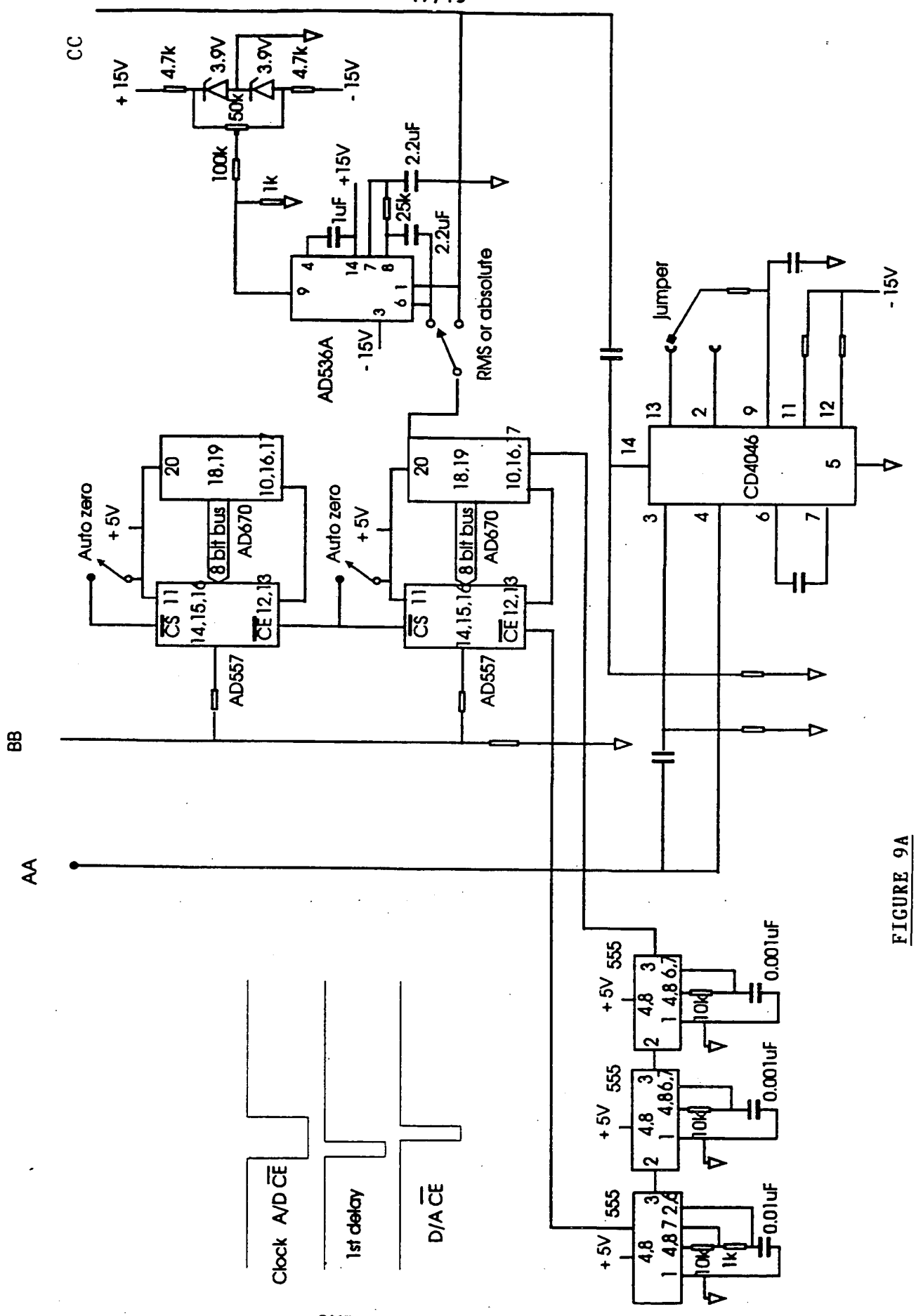


FIGURE 9A

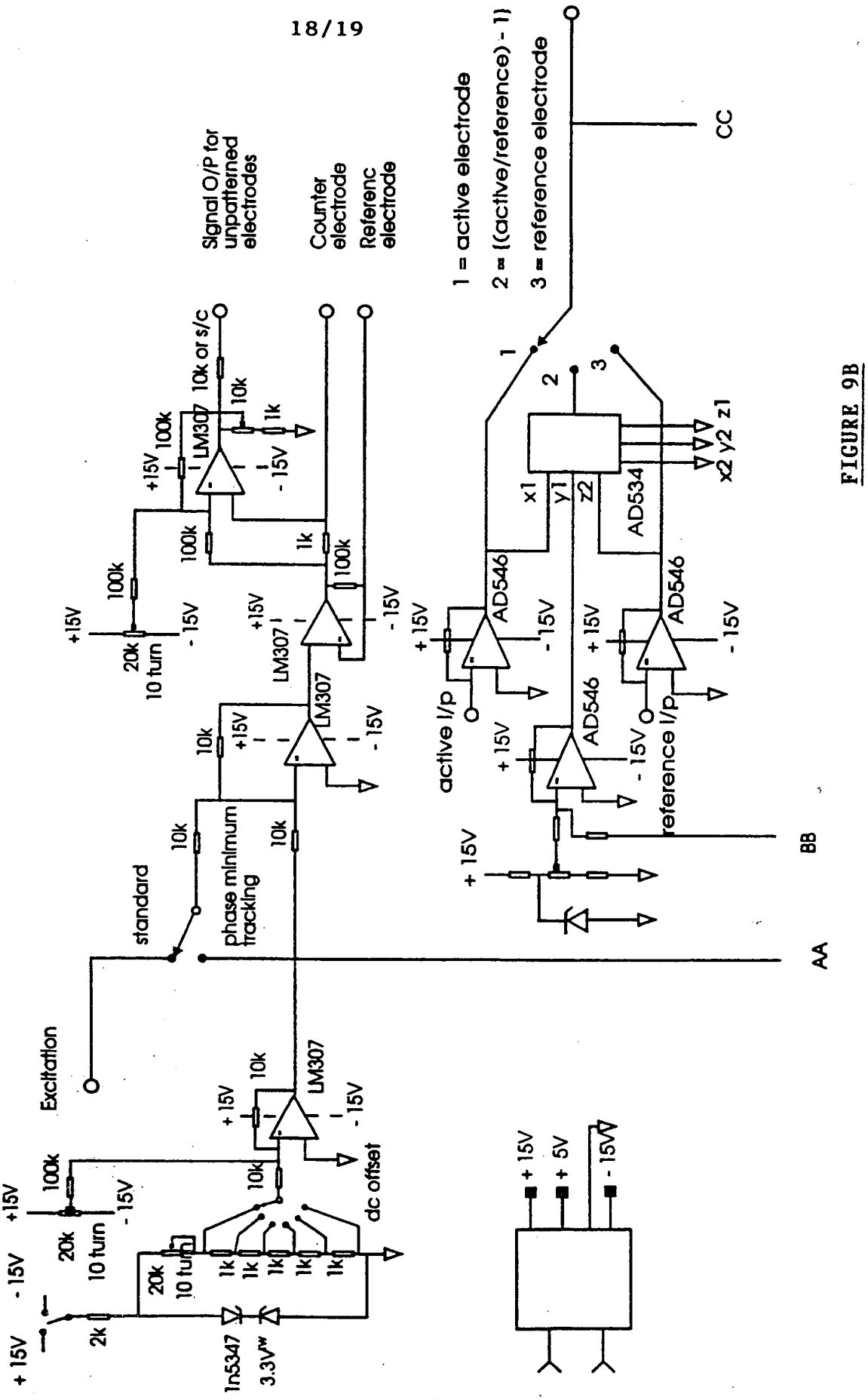


FIGURE 9B

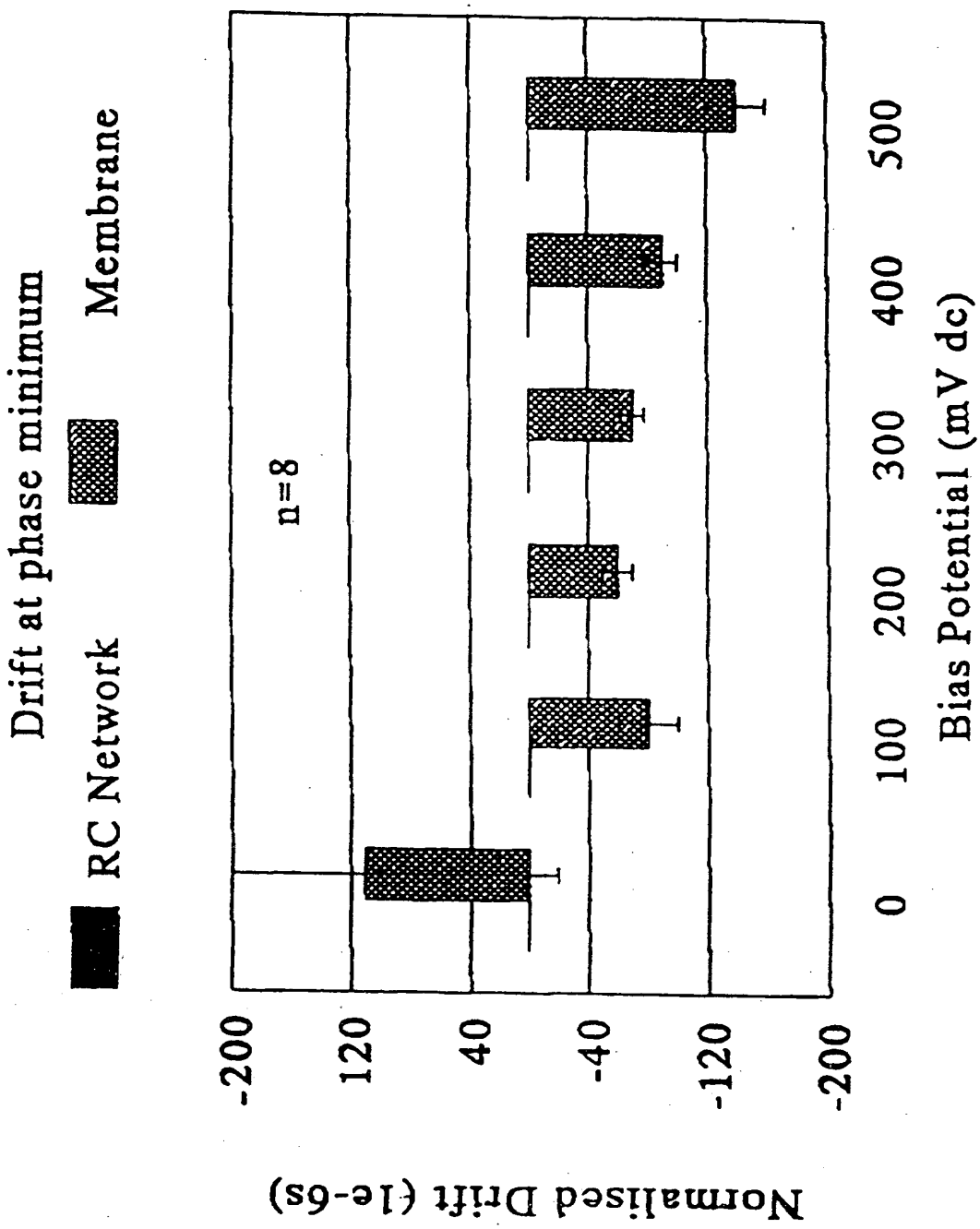


FIGURE 10

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00304

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: G01N 27/333 27/327 27/403

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC G01N 27/333 27/327 27/403 27/40 27/30

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU : IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT: membrane: ionophores: lipid: offset:
JAPIO: membrane: ionophores: lipid: offset:

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5368712 A (TOMICH et al) 29 November 1994 abstract	1, 19
A	WO 87/00168 A (WILLIS) 15 January 1987 abstract	1, 19
A	WO 92/17788 A (AUSTRALIAN MEMBRANE AND BIOTECHNOLOGY RESEARCH INSTITUTE) 15 October 1992 abstract	1, 19

☐ Further documents are listed in the continuation of Box C

☒ See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 August 1996	16 AUG 1996
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer Z. STANOJEVIC Telephone No.: (06) 283 2168

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00304

Box 1 Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 27-36
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Apparatus claims are appended to method claims and vice-versa thus rendering the scope of the claims indeterminate.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No.
PCT/AU 96/00304

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
US	5368712						
WO	87/00168	AU	60000/86	EP	228456	AU	61244/86
		AU	602868	CA	1257331	EP	230449
		WO	8700286				
WO	92/17788	AU	14657/92	AU	666113	EP	639269
		US	5401378				

END OF ANNEX

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